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REVIEW AND SYNTHESES

Immunosenescence in wild animals: meta-analysis and outlook

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Abstract

Immunosenescence, the decline in immune defense with age, is an important mortality source in elderly humans but little is known of immunosenescence in wild animals. We systematically reviewed and meta-analysed evidence for age-related changes in immunity in captive and free-living populations of wild species (321 effect sizes in 62 studies across 44 species of mammals, birds and reptiles). As in humans, senescence was more evident in adaptive (acquired) than innate immune functions. Declines were evident for cell function (antibody response), the relative abundance of naïve immune cells and an *in vivo* measure of overall immune responsiveness (local response to phytohaemagglutinin injection). Inflammatory markers increased with age, similar to chronic inflammation associated with human immunosenescence. Comparisons across taxa and captive vs free-living animals were difficult due to lack of overlap in parameters and species measured. Most studies are cross-sectional, which yields biased estimates of age-effects when immune function co-varies with survival. We therefore suggest longitudinal sampling approaches, and highlight techniques from human cohort studies that can be incorporated into ecological research. We also identify avenues to address predictions from evolutionary theory and the contribution of immunosenescence to age-related increases in disease susceptibility and mortality.

Keywords

Adaptive immunity, ageing, eco-immunology, gerontology, immune senescence, inflammaging, innate immunity, life-history trade-offs, PHA, senescence, wildlife diseases.

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INTRODUCTION

Senescence, the decline in fertility and survival with advancing age (Ricklefs 2008), was initially thought to be the exclusive domain of humans and domestic animals, because animals in the wild were unlikely to live long enough to senesce. However, it is now well documented that late-life declines in performance are widespread in wild animals (Packer *et al*. 1998; Nussey *et al*. 2013; Lemaître & Gaillard 2017; Fletcher & Selman 2015). Ultimately, senescence is thought to have evolved as a result of the decline in the force of natural selection with age and/or trade-offs between early-life reproduction and late-life performance (Nussey *et al*. 2013; Lemaître *et al*. 2015). Mechanistically, the progressive decline in somatic performance with age is associated with the accumulation of cellular and molecular damage as well as cellular and mitochondrial dysfunction (López-Otín *et al*. 2013). These changes are associated with the typical aging phenotype of the elderly (Kennedy *et al*. 2014), but less is known about the proximate ageing mechanisms in wild animals. This omission is important because the potential impact of environmental variation on the rate of senescence depends on the mechanism of aging. To understand the evolution and ecology of senescence, we need a more detailed understanding of how organismal ageing manifests in natural systems, away from medical interventions, exposed to real-world environmental challenges, in the context of natural selection.

Throughout life, the immune system is critical for controlling infections and diseases, and functioning of the immune system is therefore important for organizmal health and viability. Challenges to the immune system are diverse, and, perhaps for this reason, the immune system is complicated and comprises many components (for an overview of the main immune cell types and their functions, see Table 1). In humans, numerous epidemiological studies have demonstrated profound changes in immune function with age, implying that functional immune defense declines in old age (Larbi *et al*. 2008; Goronzy & Weyand 2013; Shaw *et al*. 2013; Giefing-Kröll *et al*. 2015; Simon *et al*. 2015; Bauer & la De Fuente 2016). Older individuals are at higher risk of developing acute viral and bacterial infections; those infections are of greater severity; vaccines are less effective; chronic infections can resurface; and there is a higher mortality risk from infections in the elderly (see also Table 1 for details). A decline in function of the immune system with age (immunosenescence) is therefore potentially an important contributor to senescence in wild populations (Promislow *et al*. 2006).

Contrary to the wealth of information on human immunosenescence, the importance of immunosenescence in wild animals is not well documented. However, understanding

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immunosenescence is important to understand ecology and evolution of senescence and lifespan: if older age classes are more susceptible to infectious diseases, an increase in disease prevalence would disproportionally increase mortality of older age classes. Since mathematical models show that only age-specific mortality can explain the evolution of senescence (Moorad *et al*. 2019), age-dependent changes in mortality rate due to immunosenescence may therefore be important to explain the evolution of shortened life span and accelerated senescence. In addition, investigating evolutionary conserved patterns of immunosenescence in wild animals will not only reveal factors that influence how immune function changes with age, but may also shed light on the causes of the often overactive immune systems of humans (allergies, autoimmune diseases, Maizels & Nussey 2013). It is, however, important to realize that not all age-associated immune changes are necessarily detrimental to the individual (Fulop *et al*. 2018). Some of the observed changes could alternatively be perceived as immune–remodelling, a change in some parts of the immune system to offset loss of function in another part (Fulop *et al*. 2018) or an adaptation to the changed demands placed upon the immune system in an older individual. Even when detrimental to individual health and survival, from the perspective of senescence as an evolved process, immunosenescence may represent an adaptive trade-off resulting from increased investment in reproduction in early life (Lemaître *et al*. 2015). To what extent declines in immune function with age are adaptive is best investigated in natural systems that provide an evolutionarily and ecologically relevant context in which to study (immuno) senescence (cf. Ricklefs 2010).

The primary aim of our study is to apply a systematic meta-analytical approach to evaluate the evidence for the occurrence of immunosenescence in wild vertebrates (amniotes). We include free-living and captive populations for comparison to gain insight into what extent environmental variation can explain variation in immunosenescence (for example, actuarial senescence patterns differ between wild and captive (zoo) populations; Tidière *et al*. 2016). Senescence is the result of an increase in susceptibility to environmental and physiological challenges with age (Medawar 1952), including infections and disease, which can be difficult to simulate in captivity (Pedersen & Babayan 2011). Therefore, the effect of immunosenescence may be stronger in the wild, where an unchecked infection can affect the ability to acquire and defend resources, and in this way have a cascading effect on physiological state that accelerates senescence (Verhulst *et al*. 2014). We also assess whether longer-lived animals show less evidence of immunosenescence, as predicted by life-history theory (Nussey *et al*. 2013; Lemaître *et al*. 2015) and observed for actuarial senescence (Jones *et al*. 2008; Ricklefs 2010). Finally, we compare evidence for immunosenescence between males and females, since sex differences in lifespan and the rate of actuarial senescence are prevalent (Brooks & Garratt 2017).

Immunosenescence in humans is used as a frame of reference. Vertebrates share a complex two-armed immune system consisting of innate (natural) and adaptive (clonotypic, or memory-based) components (see Table 1). These appear to be modulated differently in elderly humans, with strong evidence for a decline in the adaptive system, but less pronounced, or absence of, immunosenescence in innate immune aspects (Table 1). We quantify whether immunosenescence in wild animals follow this general pattern, and complement this with fine-grained analyses at the level of individual immune

Table 1 The vertebrate immune system main cell types, their functions and senescent changes in humans

Main cell types and functions	Senescent changes in humans
(1) Adaptive (clonotypic) immune system	
Cell-mediated. Naïve T cells mature in the thymus. Activated naïve T cells differentiate into cytotoxic or killer (CD8+) T cells (that can directly kill infected cells), T-helper cells (Th, CD4+) that can be pro-inflammatory (Th1) or anti-inflammatory (Th2), and immune-suppressive regulatory T cells (T_{regs}).	Decline in numbers of naïve T-cells, especially naïve killer T-cells Increase in number of memory T-cells, and T_{regs}[1,2] Increase in, and reversal of, CD4+ to CD8+ T-cell ratio[3] Reduced cell function[3]
Humoral. Activated B cells produce antibodies (immunoglobulins), proteins that recognise and neutralise pathogens.	Cell numbers mostly preserved. Decline in naïve cells, but less pronounced than for T cells; Reduced antibody production to novel antigens[4]
(2) Innate immune system	
Phagocytic cells, such as macrophages (monocytes) and granulocytes (neutrophils, basophils and eosinophils) carry Toll-like receptors (TLR) that recognise a limited number of targets shared by invaders. Activated cells produce cytokines to trigger the acute phase response	Number and phagocytic capacity of granulocytes (neutrophils) appears to be well preserved Monocytes/macrophages may show reduced function (cytokine production) and reduced TLR expression[5]
Natural killer (NK) cells are activated by lack of self-antigens on the surface of infected and tumour cells, which are subsequently lysed	Reduced function (e.g. cytokine production) per cell; Increased cell numbers; Total function is maintained[2]
Dendritic cells carry TLR, and present antigens to T-cells, bridging adaptive and innate immunity	Reduced TLR-induced cytokine production; Increased basal cytokine production[6]

The immune system can be divided into two systems that are functionally interconnected. The ***innate (natural) immune system*** provides immediate protection by rapidly recognising and eliminating pathogens identified by a limited number of targets shared by many invaders. Long-term immunological memory to specific antigens is central to the ***adaptive (clonotypic) immune system***, also known as the acquired or specific immune system. The adaptive response is based on an enormous repertoire of naïve cells that each express unique receptors and recognise a huge variety of antigens. When binding a target foreign antigen, cells undergo clonal expansion. Upon completion of the immune response, most cells are destroyed, whereby a population of high affinity memory cells is retained. Upon re-exposure, these generate a fast, efficient, memory (secondary) antibody response. ***Cytokines*** such as interleukins (ILs) are messenger molecules between immune cells, connecting both systems, regulating immune and inflammatory responses
Adapted from: [1](Gruver *et al*. 2007); [2](Müller *et al*. 2013); [3](Bauer & la De Fuente 2016); [4](Frasca *et al*. 2011); [5](Panda *et al*. 2009); [6](Shaw *et al*. 2013).

parameters. Additionally, we assess evidence for systemic inflammation, since chronic activation of the inflammatory system appears to be a pervasive aspect of human ageing, intricately linked to immunosenescence ('inflammaging'; Bruunsgaard *et al*. 2001; Fulop *et al*. 2018). We compare wild animals to humans, rather than laboratory rodents because these are not optimal models to study ecology of immunosenescence (e.g. Müller *et al*. 2013, Nikolich-Zugich & Cicin-Sain 2010): laboratory rodents are typically artificially selected to have shorter lifespans and the speed of life-history affects the rate of senescence (Jones *et al*. 2008; Ricklefs 2008). Immunosenescence in humans might thus be a better model for wild animals, and the reverse may also be true.

Finally, because we encountered various limitations and unexplored opportunities of current approaches, we identify future research directions for studying immunosenescence in wild animals.

METHODS

Literature search and classifications

We performed a systematic literature search (see PRISMA chart Fig. S1; Moher et al. 2009) for studies of age-related changes in immunity in wild amniotes because they share a very similar immune system with adaptive and innate components and similar life-history (no larval stages). We included studies that sampled wild animals from free-living and captive populations (i.e. we did not include studies of domesticated or genetically modified animals). We selected studies that measured at least one component of immunity that was compared between 'young' or 'middle-aged' and 'old' or 'geriatric' adults (i.e. we excluded comparisons with juvenile or immature individuals that represent age-related changes during immune system maturation); 5 studies of reptiles used body size as a proxy for age. All effect sizes and detailed descriptions of immune measurements were extracted by one person (KD); for details see Table S1.

In addition to analysing whether individual immune parameters ($n = 26$, with 1–50 effect sizes each) showed consistent declines, we also analysed whether declines were more likely in adaptive compared to innate immunity, as predicted from observations in humans (for details on immune parameters and senescent changes in humans, see Table 1). We classified each immune measurement into the different components of the immune system as: 'adaptive' ($n = 155$ effects; including antibody production, T-cell populations, circulating antibody levels, T-cell proliferation); 'innate' ($n = 95$ effects; these included natural antibodies, complement lytic activity, bactericidal activity); details on all classifications are given in Table S1. Some measurements integrated aspects of both 'adaptive and innate' immune function at the same time, and these were defined as adaptive + innate ($n = 42$ effect sizes; local T-cell mediated inflammation response (swelling) to subcutaneous injection of phytohaemagglutinin (*in vivo* PHA response; no data available for mammals), total white blood cell count (WBC), total immunoglobulin). Additionally, we classified immune measurements into broad functional categories: humoral immunity (antibody response, circulating antibodies); lytic capacity (killing or lytic activity of blood and plasma, lysozyme); *in vivo* PHA response; cell function (*in vitro* responses to non-pathogenic mitogens (PHA; Pokeweed mitogen (PWM); Concanavalin A (ConA), lipopolysaccharide (LPS), cell-mediated lysis or phagocytosis); cell count (WBC, counts for cell types); and cell profile (memory, naïve, immune-suppressive regulatory T-cells (T_{regs}); data only available for mammals); changes in relative abundance (%) of other immune cell types were not included, as there are no linear predictions on what constitutes senescence. Finally, in each of these three analyses we included inflammatory markers ($n = 29$ effect sizes; cytokines, haptoglobin, Amyloid A, alpha- and beta-globulins). For all immune markers, we considered that a decline with age reflects a decline in function, except for memory cells ($N = 13$ effect sizes) and regulatory T-cells ($N = 8$ effect sizes), where we considered increases to reflect a decline in function (for rationale see Table 1); these effect sizes were therefore multiplied by -1. Likewise, for inflammatory markers ($N = 24$ effect sizes), we considered increases to reflect a decrease in function (increase in inflammation), and effect sizes were also multiplied by -1; anti-inflammatory markers were assumed to reflect reduced inflammation, and effect sizes were not multiplied. Assigning negative effect signs to those parameters where an increase is indicative of loss of function means that effects, and their direction, can be interpreted in a uniform manner and enabled us to combine immune and inflammatory and anti-inflammatory markers in one single meta-analysis. All classifications were made from detailed descriptions of immune measurements by one person (AP). For a full list of studies, species, immune parameter details and categories, estimates and errors, see Table S1.

Meta-analysis

Effect sizes (F-values, t-values, P-values, Chi^2, differences between means, etc.) together with the direction of the effects (as indicated in the text or in figures) were converted into r values and further transformed into Fisher's Zr values to meet expectations of normality (r values are bounded between -1 and 1); Zr values and their SEs were computed following equations in (Nakagawa & Cuthill 2007). All analyses were carried out on Zr values, but we only report back-transformed r values for ease of interpretation.

Statistical tests assessing age-related changes in immune parameters were heterogeneous; when discrete age cohorts were compared, we excluded comparisons with young or immature animals; when those comparisons were not reported in the original study, we extracted the effect sizes from the figures provided whenever possible; when age-related changes were assessed based on age as a continuous covariate we used the statistics of the linear age trend as effect sizes; in a subset ($N = 24$ effects), changes with age were nonlinear and models included a quadratic component that indicated a change in the age trajectory of immune parameters for older individuals. In those cases, we only used the effects associated with the quadratic components in our meta-analysis; excluding these 24 effects altogether did not alter the conclusions (Fig. S2). For 15 effects (9 studies), we could not extract the relevant

information to compute standardized effect size values (mainly due to lack of information on the direction of non-significant results) and this information could not be obtained from the authors. To avoid bias by excluding non-significant effects, we conservatively replaced those effects with 0 (i.e. no age effects on immune parameters).

We used a mixed-model meta-analytical approach, using the R packages MCMCglmm (Hadfield 2010) and metafor (Viechtbauer 2010). MCMCglmm was used to run the main meta-analyses which accounted for phylogenetic relatedness and pseudo-replication within species (multiple estimates were obtained for most species) by including species identity and phylogenetic relatedness (the inverse phylogenetic covariance matrix) as random factors. We chose not to include the random factor study identity because 12 out of 44 species (27%) were represented by a single study in the sample and hence study identity was heavily confounded with species identity. The phylogeny (Hinchliff *et al.* 2015) used to compute a phylogenetic covariance matrix was constructed using the package 'rotl' (Michonneau *et al.* 2016), and branch lengths were set following Grafen's method (Fig. 1). Fixed effects included: immune parameter (26 levels, see Table S1 and Fig. 2), components of the immune system (the 26 immune parameters grouped into four categories: adaptive, innate, adaptive + innate, inflammatory) or broad immune categories (7 levels: cell profile, function and count, *in vivo* PHA response, inflammation, lytic capacity, and humoral), type of study (longitudinal or cross-sectional), sex (effects obtained on females, males or both), whether the study was carried out on wild or captive animals, and, finally, we also tested for a longevity effect (scaled) to determine whether declines in immune function were more or less marked in species along the slow–fast life history continuum (Jones *et al.* 2008; Ricklefs 2008). Longevity was derived using maximum lifespan estimates from the AnAge database of animal ageing and longevity (Tacutu *et al.* 2018), from wild populations where possible (n = 22 species); where no estimate for a species was available, we used the mean for the estimates for the genus (n = 3).

We ran nine models, denoted m1 through m9, which included the intercept-only model (m1), and eight additional models each including one fixed effect/moderator at a time: Class (m2), sex (m3), wild/captive (m4), longitudinal/cross-sectional (m5), longevity (m6), components of the immune system (m7), broad immune categories (m8) and immune parameters (m9). In addition, we ran three full models each including one of the classifications of the immune variables (components of the immune system, broad immune categories and immune parameters) and all other fixed effects.

We used parameter expanded priors for the random effects (list(V = 1, nu = 1, alpha.mu = 0, alpha.V = 1000)), inverse gamma priors (list(V = 1, n = 0.002)) for the residuals and normal distributions centred on zero with large variances as fixed effects priors (default prior in MCMCglmm). These priors were chosen to improve model convergence while being minimally informative (random effects) or completely uninformative (fixed effects). Models were run across 5005000 iterations with thin of 5000 and a burn in of 5000 which resulted in a posterior sample of 1000. These values were determined based on model convergence and autocorrelation levels assessed through trace graphs and autocorrelation plots. We further tested whether models converged on the same results by running each model in Fig. 2 twice and comparing them using the Gelman–Rubin test in the package coda (Plummer *et al.* 2006). In all cases, the potential scale reduction factor was lower than 1.03, which is below the threshold of 1.1 indicating model convergence. For each model, we computed marginal (fixed effects) and conditional (fixed and random effects) R^2 values following Nakagawa & Schielzeth (2010).

Publication bias was assessed based on visual inspection of funnel plots of 'meta-analytic' residuals in each model (see Fig. S3 for funnel plots), Egger's test on the meta-analytic residuals (Nakagawa & Santos 2012) and trim and fill methods (Duval & Tweedie 2000), 'trimfill' function using method 'R0' in metafor (Viechtbauer 2010). Total heterogeneity, and heterogeneity due to phylogeny, study and species identity were computed following (Nakagawa & Santos 2012). All analyses were carried out within the R (v. 3.4.0) statistical environment (R Core Team 2017).

RESULTS

Our data set of 62 studies involved 321 effect sizes from 44 species, mostly birds (n = 20 species) and mammals (n = 19) with only 5 reptiles (for details see Fig. 1, see Table S1 for details on all studies and Tables S2 and S3 for details of the main statistical models m1-m9). There were 35 studies from the wild (49 effects from birds, 54 from mammals, and 26 from reptiles), and 28 studies from captive animals (61 effects from birds, 131 from mammals and 1 from a reptile; one study reported effects for wild and captive species). No species was studied both in the wild and in captivity. There was limited overlap in immune parameters measured in the wild and in captivity, and even less so across the Classes. The majority (94%) of effects were from cross-sectional analyses, comparing immune parameters between age-classes or across a range of ages in known-age individuals.

The intercept-only model revealed that across all studies there was a small, non-significant, negative effect (m1 in Fig. 2, Table S2 and S3; mean: 95% CI = −0.139, −0.368–0.102, P = 0.19), indicating no statistically significant change in immune function with age across all measurements combined. Total heterogeneity was high (0.96, 95% credible interval (CI) = 0.95–0.97) while heterogeneity due to random effect species identity (0.04, 95% CI = 0.00–0.12) and phylogeny (0.08, 95% CI = 0.00–0.25) were low.

Computing effects separately for each taxonomic class revealed declines in immunity with age for mammals and birds and small increases in reptiles, but none of these effects were statistically significant (m2 in Fig. 2, Table S2 and S3). We found no evidence for sex-specific effects: estimates of changes in immunity with age were indistinguishable between males, females or sexes combined (m3 in Fig. 2, Table S2 and S3). Effects derived from animals in captivity indicated a stronger decline in immunity with age compared to those obtained in the wild, but these differences were not statistically significant (m4 in Fig. 2, Table S2 and S3), and not upheld in more complex models (see below). Similarly, there were no obvious differences between cross-sectional or

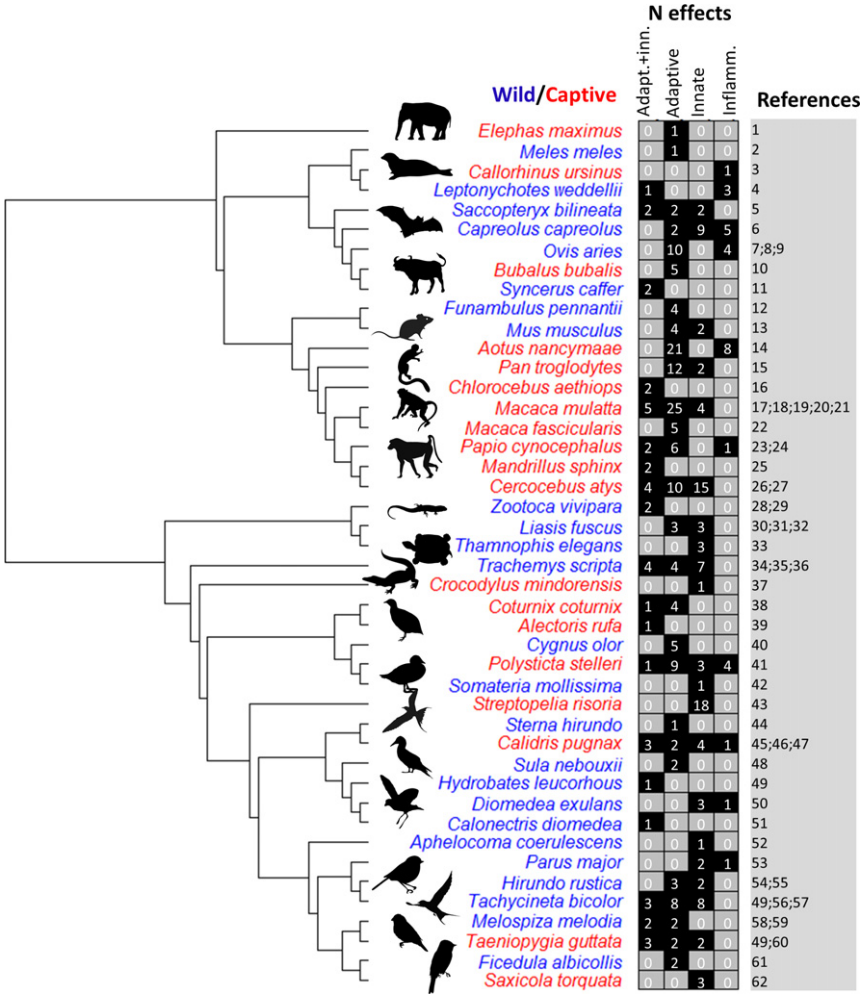

Figure 1 Graphical summary of the phylogenetic distribution of species contributing statistical effects addressing immunosenescence in non-domesticated animals. Depicted are phylogenetic relatedness on the left, scientific names for all species included (blue = data collected in wild population, red = data collected in captive population), and for each species the number of effects in each main component of the immune system and the relevant references. References: 1: (Lindsay et al. 2010); 2: (Beirne et al. 2016); 3: (Mazzaro et al. 2004); 4: (Mellish et al. 2011); 5: (Schneeberger et al. 2014); 6: (Cheynel et al. 2017); 7: (Graham et al. 2010); 8: (Nussey et al. 2012); 9: (Watson et al. 2016); 10: (Grandoni et al. 2017); 11: (Ezenwa & Jolles 2008); 12: (Ahmad et al. 2012); 13: (Abolins et al. 2018); 14: (Nehete et al. 2017a); 15: (Nehete et al. 2017b); 16: {Castro:2015hz}; 17: (Cicin-Sain et al. 2007); 18: (Čičin-Šain et al. 2010); 19: (Coe & Ershler 2001); 20: (Coe et al. 2012); 21: (Ershler et al. 1988): 22: (Higashino et al. 2011): 23: (Eichberg et al. 1981); 24: (Jayashankar et al. 2003); 25: (Setchell et al. 2006); 26: (Chakrabarti et al. 2000); 27: (Sharma et al. 2014); 28: (Massot et al. 2011); 29: (Richard et al. 2012); 30: (Madsen et al. 2007); 31: (Ujvari & Madsen 2006); 32: (Ujvari & Madsen 2011); 33: (Sparkman & Palacios 2009); 34: (Zimmerman et al. 2010); 35: (Zimmerman et al. 2013); 36: (Zimmerman et al. 2017); 37: (Groffen et al. 2013); 38: (Lavoie et al. 2007); 39: (Alonso-Alvarez et al. 2009); 40: (Hill et al. 2016); 41: (Counihan & Hollmén 2018); 42: (Neggazi et al. 2016); 43: (Terrón et al. 2004); 44: (Apanius & Nisbet 2003); 45: (Lozano & Lank 2003); 46: (Lozano & Lank 2004); 47: (Nebel et al. 2013); 48: (Torres & Velando 2007); 49: (Haussmann et al. 2005); 50: (Lecomte et al. 2010); 51: (Catry et al. 2011); 52: (Wilcoxen et al. 2010); 53: (Vermeulen et al. 2017); 54: (Møller & Haussy 2007); 55: (Saino et al. 2003); 56: (Palacios et al. 2007); 57: (Palacios et al. 2011); 58: (Reid et al. 2003); 59: (Reid et al. 2007); 60: (Noreen et al. 2011); 61: (Cichoń et al. 2003); 62: (Tieleman et al. 2010). Silhouettes depicting selected taxa obtained from phylopic.org.

longitudinal effects (m5 in Fig. 2, Table S2 and S3). It should be noted however, that there were only five longitudinal studies and there was limited overlap in immune parameters measured across Classes and in the wild or captivity, making these results quite preliminary. Longer-lived species tended to show less evidence for immunosenescence (a positive effect for longevity; m6 in Fig. 2, Table S2 and S3), but also this effect was far from being statistically significant.

We did find some differences in effects when we grouped immune measurements into different categories, allocating each effect to the corresponding component of the immune system (adaptive, innate, adaptive + innate combined or inflammation components). This revealed negative effects consistent with immunosenescence for adaptive, combined and inflammatory markers, but not for innate immunity (m7 in Fig. 2, Table S2 and S3). While these effects all have 95% CIs that overlap zero, pairwise contrasts between immune categories revealed statistically significant differences between innate immunity and each of the other three levels (Fig. 2, m7). More detailed classifications into broad immune

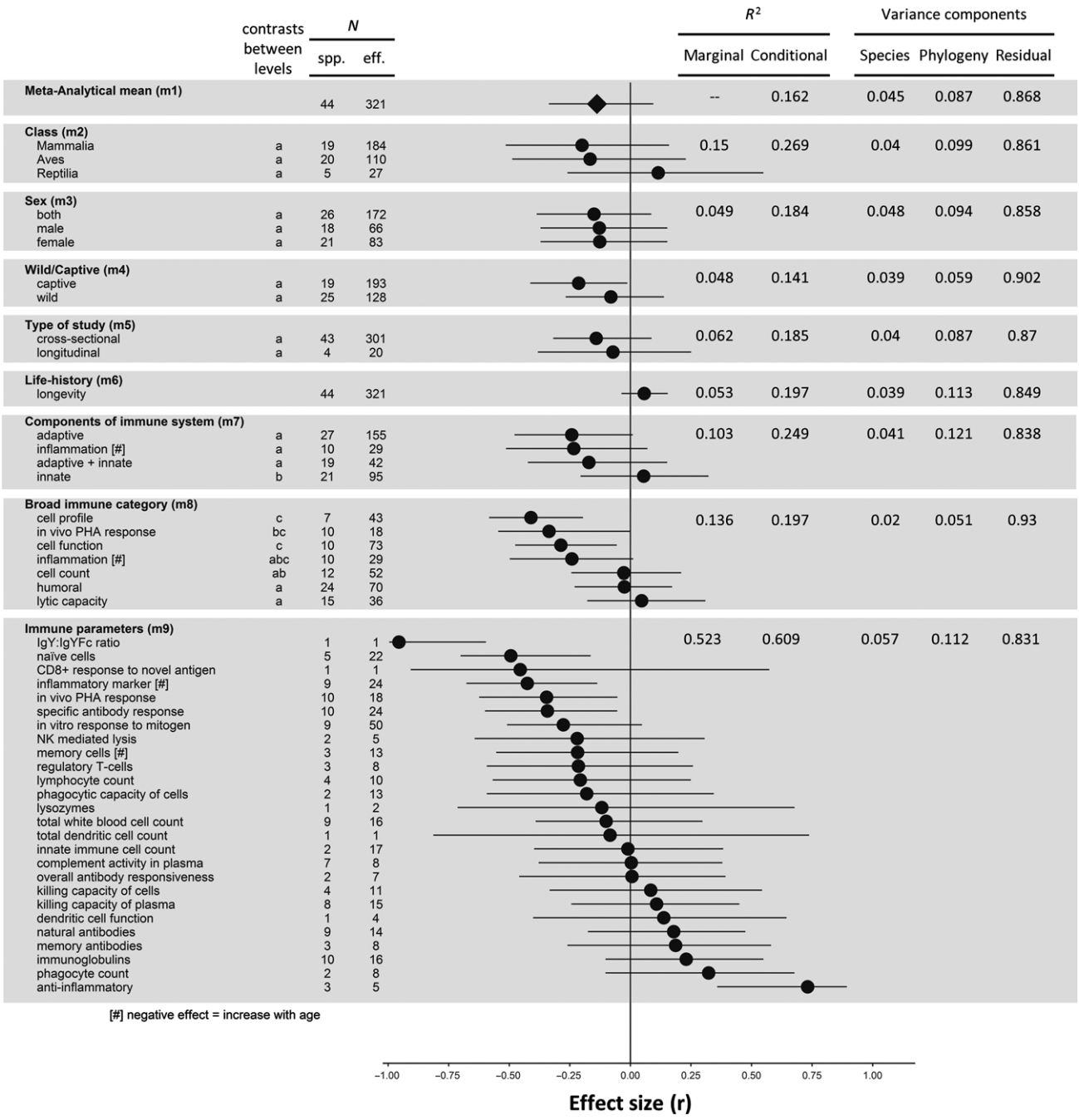

Figure 2 A summary of the evidence for immunosenescence in free-living and captive wild animals, whereby negative effects are consistent with decline in immune function with age. Forest plot depicting the overall meta-analytical mean (Pearsons' r, and 95% credible intervals) and the effects of moderators on this mean (Class, sex, wild/captive study, cross-sectional or longitudinal study, life-history (maximum lifespan)). Additionally, changes in immune function with age were analysed separated at three different levels of classification: by components of the immune system (adaptive, innate, adaptive and innate combined), across broad functional categories, and separate immune parameters. Numbers reflect number of effects sizes and species for each effect; shared letters indicate effects that are not statistically significantly different (this was not done for m9 for ease of interpretation, see Table S2 for this information). Note that the sign of the effect sizes for inflammation, inflammatory markers, memory and regulatory cells was inverted, because increases in these variables are consistent with immunosenescence; negative effect sizes therefore reflect increases in these parameters (for rationale see Table 1). Full information on effect sizes, 95%CI, and sample sizes is provided in Table S2.

categories and immune parameters pointed to further differences. When we classified effects into broad immune categories (m8 in Fig. 2, Table S2 and S3), there was evidence of immunosenescence in immune cell profile, cell function, in vivo PHA responses, and also negative effects for inflammation (note that the negative sign here indicates greater inflammation, consistent with inflammaging). In the analysis at the level of immune parameters (m9 in Fig. 2, Table S2 and S3), these conclusions were largely upheld. It appeared that senescent changes in immune cell profile were

mostly due to declines in the number of naïve cells, with less pronounced increases in memory cells and regulatory cells (m9 in Fig. 2, Table S2 and S3). Senescent increases in inflammation were due to increases in inflammatory markers (whereby few studies determined anti-inflammatory cytokines). Specific antibody responses (keystone of adaptive immunity) clearly declined with age, while *in vitro* cell responses to mitogens (key component of cell function) also decreased with age, but not significant. There were other significant effects in this analysis, but some of these are based on extremely small sample sizes and restricted to few species (m9 in Fig. 1, Table S2 and S3) and hence tell us little about the generality of these effects.

Given that there was variation in effects across different categorisations of immune measures, we further tested whether there were effects of the other moderators (sex, wild/captive, longitudinal/cross-sectional studies, and longevity) once variation across immune categories was accounted for. We found no statistically significant effects of these moderators once we accounted for differences across immune categories (Table S4–S6).

Across all models in Fig. 2, the variation accounted for by random factors phylogeny (0.05–0.11, Table S3) and species identity (0.02–0.05, Table S3) was small (cf. Senior et al. 2016). Taken together, these results suggest that changes in immune function with age vary between immune system components, between different immune categories and parameters, but also that there is substantial unexplained variation in effect sizes. Finally, we found little evidence of publication bias in terms of funnel plot asymmetry of meta-analytical residuals as revealed by plot inspection (Fig. S3). Results from Egger's test suggested some degree of asymmetry for some (m1, m2, m3, m6, m6; Table S3), but not for other models, in particular those where moderators accounted for differences in effects across components of the immune system (m7), immune categories (m8) or immune parameters (m9, Table S3). Similarly, the trim and fill procedure did not reveal funnel plot asymmetry across any model (Table S3).

DISCUSSION

Extrapolating from the patterns observed in humans, the predictions for immunosenescence in wildlife are as follows: decline in the overall immune function, mostly as a result of declines in adaptive immunity, with less pronounced decline or no change in innate immunity, and increases in inflammatory markers (inflammaging) (Table 1). Our results suggest that wild animals do indeed show senescence in some aspects of overall immunity, mostly in the adaptive immune system, with evidence for inflammaging, and no evidence for senescence in innate immune parameters (Fig. 2). However, the evidence is not conclusive and there is substantial heterogeneity between studies and species.

The observed greater disease susceptibility of elderly humans is mostly attributed to declines in aspects of adaptive (also called acquired, specific, clonotypic or memory-based) immunity (Table 1). However, T-cell memory developed earlier in life generally functions well into old age, formation of specific antibodies to novel infections and generation of novel immune responses is significantly impaired later in life (Haynes & Maue 2009). These changes are a result of reduced cell function and depletion of naïve cells due to accumulated exposure to antigens ('life-long antigenic load') (Lin *et al.* 2016). Given the generality of such mechanisms across taxa (Shanley *et al.* 2009), we predicted a decline with age in adaptive immune function. Indeed, the clearest changes we identified in older animals all relate to adaptive immunity: a change in cell profile as a result of a strong decline in naïve cells (Fig. 2), in addition to less pronounced increases in memory and regulatory cells; a decline in specific antibody responses; reduced cell function (mostly *in vitro* cell responses to novel antigens/mitogens); all suggesting a similar decline in adaptive immune function with age as seen in humans. Also, in agreement with predictions is that the *in vivo* PHA response showed an overall decline with age. While this assay is not used in humans, it is a broad parameter that includes aspects of innate and adaptive immune responses. We also found an increase in circulating inflammatory markers, consistent with 'inflammaging', the state of chronic low-grade inflammation closely associated with the dysregulation and lower efficiency of the immune system, particularly the innate immune responses, in the elderly (Michaud *et al.* 2013; Shaw *et al.* 2013). The fact that anti-inflammatory markers also seem to increase, rather than decrease, with age (positive effect in m9 in Fig. 2; in 3 species of mammal) is in agreement with the emerging realisation that inflammaging might reflect an overall activation of the inflammatory system (Morrisette-Thomas *et al.* 2014). Contrary to predictions from sex-differences in the rate of aging, we found no evidence for differences in immunosenescence between the sexes (Fig. 2), similar to a recent meta-analysis showing no sex differences in immunity overall (Kelly *et al.* 2018).

Our comparisons of immunosenescence between captive and wild animals, as well as among taxonomic classes, were limited by methodological differences and biases among studies. Although not significant, the age-related decline in immunity for captive species was greater than in wild species, which is contrary to our prediction of a stronger decline in immune function with age in wild animals, since captive animals live longer (most clearly demonstrated for short-lived mammals in zoos, Tidière *et al.* 2016). Possibly, some individuals in captivity might reach (very old) age rarely observed in the wild, and if their immune status is strongly senescent (but not lethal in protected conditions), this may potentially explain why immunosenescence effects tended to be stronger in captive populations. On the other hand, captive animals may experience suboptimal conditions, such as inappropriate social conditions, (micro)nutrient deficiencies, stressful temperature or light regimes, or space constraints, that might aggravate the symptoms of senescence. However, no single species was examined in captivity and in the wild, and mostly different immune assays were used in both types of study. Therefore, we cannot confidently conclude what effect the captive environment has on immunosenescence. Similarly, statements on the differences between the taxonomic classes are hampered by the fact that all but 1 effect from reptiles were in the wild, whereas a greater proportion of mammals and birds were studied in captivity. Furthermore, of the 26 immune

parameters, only 7 were measured in all 3 Classes while 12 were unique to 1 Class. Currently, we have no evidence of large differences in immunosenescence between mammals and birds, but further research is needed. In addition to such biases, the vast majority of studies are cross-sectional comparisons between age cohorts, which reduces the strength of all our inferences.

We consider the lack of longitudinal studies the greatest limitation on our understanding of the importance of immunosenescence in wild populations. Perhaps surprisingly, this is a problem shared with human studies (Lin *et al.* 2016). The limitations of cross-sectional studies are substantial. Senescence is a within-individual process, whereas cross-sectional patterns represent a combination of within-individual changes and changes in population composition (van de Pol & Verhulst 2006). Importantly, populations show heterogeneity in mortality risk; some individuals are more susceptible to death than others (Vaupel *et al.* 1979). The selective disappearance of these higher-risk individuals (also referred to as demographic selection) causes the characteristics of cohorts to change over time, irrespective of within-individual changes. Two longitudinal studies of immunosenescence in wild animals highlight the potential pitfalls of relying on cross-sectional results. Older female Soay sheep (*Ovis aries*) display higher antibody production, but this cross-sectional pattern could be attributed to selective disappearance of individuals with lower values (Graham *et al.* 2010). A second longitudinal study, in European badgers (*Meles meles*), showed that the *in vitro* response to a T- and B-cell mitogen was lower in older individuals, declined with age within individuals, but also that weakly responding individuals selectively disappeared from the population over time (Beirne *et al.* 2016). Such selective disappearance of individuals with lower responses can mask senescent patterns at the population (cross-sectional) level, as has been demonstrated for demographic studies of humans (Vaupel *et al.* 1979; Vaupel & Yashin 1985) – an issue that is inevitable if trait values of interest correlate with survival. For example, if individuals with less active innate immunity are relatively more likely to die young than those with less active adaptive immune responses, this can explain the preponderance of evidence for age-related declines in adaptive vs innate immunity (Table 1), even if both systems senesce similarly. It should be noted that in this context, in addition to differential mortality, selective disappearance may also mean selective recapture probability. For instance, innate immunity can covary with behavioural type and risk-taking (Zylberberg *et al.* 2014; Jacques-Hamilton *et al.* 2017), which can affect recapture probability, thereby generating biases in observed age-related change. Depending on the direction and magnitude of within-individual and between-individual patterns, when pooled in a cross-sectional analysis, these effects can result in significant negative, positive or neutral overall patterns. Distinguishing these two effects is therefore essential to distinguish senescence from selective disappearance.

OVERCOMING STUDY DESIGN LIMITATIONS

Longitudinal studies require the ability to repeatedly sample individual animals using appropriate techniques/assays and strong experimental designs, and we here discuss methods to achieve these aims in studies of immunosenescence in the wild.

General design considerations

When selecting immune parameters to assess their contribution to senescence, it will be important to verify how these parameters are associated with fitness. A decline in one immune trait does not necessarily reflect an overall decline in immune function, or in individual health. Furthermore, because canalisation is likely to be stronger for immune traits with larger fitness consequences, these are less likely to show senescence, i.e. the traits that show the strongest changes with age may do so because net benefits of continued allocation to these traits are low, relative to traits that change little with age (Boonekamp *et al.* 2018).

Large sampling requirements of longitudinal designs could be alleviated by incorporating Planned Missing Data Design (PMDD, Noble & Nakagawa 2018). In PMDD designs, measurements are deliberately omitted from subjects by randomly assigning them to have missing measurements or measurement occasions. Researchers can then utilise standard techniques to fill in missing data such that the data contains complete information for all variables and experimental units within the data set (discussed in (Noble & Nakagawa 2018). Sampling only subsets of subjects at each sampling point, can give subjects a 'break' from longitudinal sampling series, saving time and cost for time-consuming or expensive measurements. Such designs could even include a two-method-design approach, where subjects are assigned to two different assays that vary in value (price, measurement error, etc.), which is particularly effective for longitudinal sampling.

Immune function, can be assessed *in vivo* (inducing and subsequently measuring an immune response) or *in vitro (ex vivo)*, from a single sample, and the decision to use either type of assay has large implications. Firstly, *in vivo* studies allow testing of whole-organism-level immune functions, possibly more likely related to fitness, whereas *in vitro* techniques can be more targeted to specific immune components. However, *in vivo* techniques require multiple captures at each age: a first capture to induce an immune response, after which individuals need to be recaptured within a specific timeframe to assess the response, while *in vitro*/*ex vivo* techniques require only a single sample at each age, and are therefore less challenging in wild animals. More importantly though, *in vivo* measures of immune function can affect the animal's state, and thereby exert carry-over effects that bias measurements at later ages. Such carry-over effects can arise in different ways, and here we suggest various approaches how they could be avoided before focussing on potential approaches to advance *in vitro* measurements.

Longitudinal sampling of in vivo immune function

Measurement of responsiveness to *in vivo* immune challenges with non-replicating antigens involves first a primary response, followed by the development of an immunological memory that enhances later (memory) responses to the same

antigen, inherently biasing estimates of age effects. This can in theory be overcome by including only second and subsequent responses to investigate changes in the memory response only. However, this is perhaps not optimal, given that the strongest expectation of immunosenescence is for the primary response to novel antigens (Table 1), and further development of memory effects with subsequent immunisations is difficult to exclude with confidence. An alternative potential solution is to use different antigens at different ages in a balanced design, assuming that antigens are sufficiently different so that cross-reactions are negligible. For both approaches, it needs to be verified that effects of previous immunisations have subsided. Verifying this may not be straightforward, because immune responses may be costly (reviews in Demas *et al.* 2011; Hasselquist & Nilsson 2012), for example through oxidative damage (Bertrand *et al.* 2006) or demands for specific nutrients (review in Hasselquist & Nilsson 2012). Thus, repeated 'measurements' of immune function may alter survival probability, even if harmless antigens are used (Hanssen *et al.* 2004). Given the ongoing uncertainty about the costs of immune responses, how important or pervasive this problem may be cannot be evaluated at this time, but it certainly must be taken into consideration. Another problem with a repeated immunisation approach is that individuals may respond by changing their behaviour, reducing their exposure to pathogens, which may affect their state in multiple ways. Lastly, immunisation can potentially alter allocation to the immune system in a non-specific way, changing their ability to respond (Schmid-Hempel 2003; Verhulst *et al.* 2005), which would bias estimates of age-effects.

Specific sampling strategies could be designed to address these issues with repeat immunisations. One approach is to induce and measure a primary immune response at a young age in a subset of the captured individuals of a cohort, while in other cohort members a similar primary response is induced and measured at an older age. When including only individuals that were captured at both ages when testing for an age effect this precludes a bias through selective disappearance. This approach can be extended by inducing and measuring a secondary response to the same antigen, and by inducing immune responses at a variety of ages. Even in its simplest form, this approach will require a large effort, but yield additional information on selective disappearance with respect to the immune response, and whether this depends on age, by comparing primary immune responses between individuals that did or did not survive to the next stage.

Technical approaches using single samples and *in vitro* assays

An alternative to repeated *in vivo* immune challenges is the repeated measurement of standing variation in circulating cell numbers, types and soluble fractions. Suitable assays involve various constitutive innate immune defenses (Demas *et al.* 2011; Matson *et al.* 2005) but also levels of acute phase proteins (Matson *et al.* 2012) and baseline expression of innate immune cell receptors (TLR Toll-like receptors; Martin *et al.* 2014). Another attractive alternative is to use *in vitro (ex vivo)* assays of responsiveness (e.g. antibody or cytokine production) of total cellular fraction or individual cells to stimulation by a variety of antigens or mitogens; such parameters showed quite clear declines with age (Fig. 1, m8, m9). It is important to note the difference in interpretation: measurements of circulating parameters are indicative of immune system status quo whereas *ex vivo* cell assays assesses the strength of an immune response.

Additionally, ecologists could take greater advantage of available techniques used in other disciplines. Most methods used by eco-immunologists are quite different from those used in human studies, usually less specific and technically less challenging (e.g. Matson *et al.* 2005, 2012). Partly, this is an inevitable consequence of less information on physiology and molecular biology of the study species, and lower availability of specific assays for non-model organisms. Nonetheless, greater use could be made of the wealth of information from the medical literature. In particular, critical immunosenescence indicators identified in human cohort studies would appear good target candidates for use in ecological studies. In the elderly, an informative predictor for a reduced response to vaccination appears to be the reduced numbers of naïve T cells (Ongrádi & Kövesdi 2010). Our analysis indicated that the abundance of naïve cells are also strongly sensitive to age in wild animals, and thus it seems promising to adapt flow cytometry assessment of memory/naïve cell subtypes (see Table 1); this has so far been done for monkeys and bovids (see Table S1), but such 'immunophenotyping' techniques are also available for birds (Kaiser 2014), although there can be some difficulty in identifying unequivocal markers for naïve cells (Müller *et al.* 2013). Measurements of circulating memory antibodies are not technically challenging, and recent development of a passerine specific anti-IgY, in addition to a bird and chicken-specific antibody (Fassbinder-Orth *et al.* 2016), is potentially a useful addition to the toolkit of avian immune ecologists. Within the innate system, reduced neutrophil and NK cell activity are predictive of increased mortality in old humans, while dysregulation of TLR function affects responsiveness to viral infections and vaccines (see Table 1; review in Panda *et al.* 2009), which can be measured in ecological studies (an example in birds: Martin *et al.* 2014). In addition to such relatively well established markers, immune gene expression can be studied in blood, and targeted novel analyses could be designed from published genome-wide studies of differential expression (e.g. Watson *et al.* 2017). In this regard it is noteworthy that a first transcriptional profile of changes in (immune) gene expression following a simulated bacterial challenge has been generated for a wild passerine (Meitern *et al.,* 2014). This study highlighted activation of several known antimicrobial and general (innate) immune response genes, as well as possible novel markers, indicating the possibility for development of alternative indexes for innate immune function in the near future.

A recently suggested broad-scale approach is to quantify age-related dysregulation of immune parameters. Here, dysregulation is quantified as the absolute distance of a biomarker profile from the average profile (Cohen *et al.* 2013; Cohen 2015), and high values are assumed to indicate greater dysregulation, i.e. greater deviation from the mean. In humans, dysregulation increases with age across multiple physiological systems, including immune parameters such as total white

blood cell count and proportion of neutrophils, monophils, basophils and lymphocytes (see also Table 1), and dysregulation significantly predicts mortality (Li *et al.* 2015). This approach does not yet appear to have been applied to assess immunosenescence in animals, but recent evidence showed that signatures of physiological dysregulation are conserved in primates (Dansereau *et al.* 2019) but a similar approach in a different context (birds) found no effects (Fowler *et al.* 2018). Thus, this may be a promising approach to assess immunosenescence in the wild, whereby it should be noted that relatively large sample sizes (>100) are required to reliably define the reference profile.

Inflammaging – chronic low-grade inflammation due to dysregulation of the inflammatory process - is closely linked to immunosenescence (Franceschi *et al.* 2007; Bauer & la De Fuente 2016), well-defined, and relatively easy to measure. However, only 10 studies assessed age-related change in inflammatory markers, despite substantial scope to fruitfully do so, with clear predictions and available techniques. For example, high levels of the cytokines TNF (tumor necrosis factor)-α and IL-6 levels have been identified as key inflammaging biomarkers in elderly humans (Singh & Newman 2011; Michaud *et al.* 2013) and several studies have observed that a (genetic) predisposition to weak inflammatory activity [e.g. high IL-10, low TNF-α] is beneficial for longevity, provided individuals avoid lethal infection early in life (review Shanley *et al.* 2009). Since cytokines are conserved across vertebrates, analytical methods can be adopted for non-model organisms (Zimmerman *et al.* 2014). With rapid development and price reductions of genomic technologies, thresholds for profiling gene-expression of non-model organisms are rapidly lowering (Fassbinder-Orth 2014).

EXPANDING THE SCOPE OF IMMUNOSENESCENCE RESEARCH IN WILD ANIMALS

The onset and progression of senescence vary dramatically among species, populations, and individuals. Although the aging process within an individual may seem maladaptive, the prevailing view is that senescence is an evolved process, and its pattern and process can be explained by evolutionary theory (Hamilton 1966). Studies of wild animals are ideal for testing evolutionary explanations for variation in senescence, in the ecological context where senescence evolved. We here tested one core prediction of evolutionary theory – that senescence is inversely correlated with the pace of life-history – by quantifying whether age-related declines in immunity are stronger in shorter-lived species (the effect of longevity). While the direction of the meta-analytic mean effect supported the prediction, this was not statistically significant, possibly due to the biases and limitations inherent in the available data (as detailed above) or because maximum longevity might not accurately represent species lifespan. Nonetheless, these results should encourage future studies into testing this hypothesis. Environmental variation likely also shapes the diversity of immunosenescence patterns across and within species. For example, immunosenescence might depend on the diversity and quantity of pathogens present or encountered in the environment (Lin *et al.* 2016). An individual's social environment, the balance of current and future reproductive opportunities, and rate or risk of predation are also likely to influence the timing or rate of immune decline, and these predictions can be tested in natural systems.

One key prediction from current evolutionary theories is that greater allocation of resources to growth and reproduction drives more rapid rates of senescence (Kirkwood & Austad 2000). Following from this prediction, we would expect growth rates and reproductive allocation earlier in life to influence patterns of immunosenescence. This idea can be addressed in longitudinal studies within populations, relating individual allocation in growth and reproduction to immune parameters later in life. An alternative way to explore this question would be through broader-scale, phylogenetic comparisons that relate growth rates and reproductive strategies to rates of immunosenescence. Such comparisons would also offer further insights into other selective pressures and ecological factors that might drive immunosenescence (Lemaître *et al.* 2015). However, addressing these fundamental questions using phylogenetic comparisons will require consideration of a broader range of species, with diverse life histories.

A better understanding of the natural history and the evolutionary dynamics of immunosenescence in wild animals would also make important contributions to other research areas, particularly due to the potential impact of immunosenescence on variation in susceptibility to parasites and diseases. Age-related patterns of infection cannot be understood without understanding age-related changes in immunity (Hill *et al.* 2016). In humans, the most important reason for the increased rate and mortality risk of infections in the elderly is the diminished function of the immune system which occurs with ageing (Ongrádi & Kövesdi 2010). If immunosenescence in wild animals is similarly important for explaining variation in infection resistance and mortality, an understanding of immunosenescence is required to understand eco-evolutionary dynamics of hosts and parasites, as well as the epidemiology and population impacts of wildlife diseases diseases (e.g. Marzal *et al.* 2016; Gervasi *et al.* 2015). For this purpose, however, we need a better understanding of how immunosenescence relates to mortality and fitness.

This, in our view, is the most important challenge for 'wild immunosenescence' research: establishing whether immunosenescence in wild animals is associated with fitness costs, and hence instrumental in the decline in fitness with age. To this end, it will be necessary to compare life-time reproductive success of individuals with differing rates of immunosenescence, in long-term studies of large numbers of individuals. Ideally, this would be combined with experimental manipulations of immune function at different ages. However, such experimental manipulations depend on the ability to manipulate immune function without side effects, which will be difficult with pharmaceuticals but may be possible using molecular tools such as RNA_i. The outcomes of this research will also make a critical contribution to our understanding of to what extent observed age-related changes in immune parameters represent dysfunction, and to what extent they are adaptive within-individual changes that maximise fitness.

CONCLUSION

Reviewing the available literature on immunosenescence in wild animals, our study revealed: (i) some evidence that immunosenescence in wild animals occurs, with the available evidence suggesting (ii) that immune function in older animals may show similarities to that of elderly humans, but also that (iii) there is substantial amounts of unexplained heterogeneity and (iv) that the available evidence is too limited to draw definitive conclusions, which we attribute in particular to the paucity of longitudinal studies.

Our analysis found some congruent patterns in immunosenescence in wildlife compared to humans (Table 1, Fig. 2). Such congruence, despite differences in ecology and life-history, in depth of mechanistic understanding, in research approaches and in immune parameters measured, suggests that immunosenescence is an evolutionarily conserved process. This shows that the eco-evolutionary roots of immunosenescence can be studied in free-living animals, and the tools for such studies are increasingly available. Moreover, we identified several immune parameters that revealed immunosenescence to occur in wild animals, notably relative numbers of naïve cells; antibody production to an immune challenge; *in vitro* responses of cells; and *in vivo* responses to PHA. These parameters may therefore be particularly useful for studies testing the predictions emerging from evolutionary aging theories. Studies of wild animals therefore provide an outstanding opportunity to inform us on the evolutionary basis and the ecological consequences of immunosenescence, which is not only critical to our understanding of ageing, but also to our broader understanding of mortality, infection and health in natural populations.

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AUTHORSHIP

AP, KD and SV designed the study, KD, AP and AA collected data, KD and SN performed the meta-analysis. AP wrote the first draft of the manuscript, and all authors contributed to revisions

DATA AVAILABILITY STATEMENT

No new data were used, the meta-analytic effect sizes extracted from the literature are included in the supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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